EFFECTS OF INTERMITTENT INJECTIONS OF LHRH ON SPECIFIC BINDING OF ^{125}I-LABELED GONADOTROPINS TO GRANULOSA AND THECA, AND CONCENTRATIONS OF STEROIDS IN SERUM AND OVARIAN FOLLICLES DURING POSTPARTUM ANOVULATION IN SUCKLED BEEF COWS[1,2]

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ABSTRACT

To examine ovarian follicular response to low-dose injections of luteinizing hormone-releasing hormone (LHRH), 32 anovulatory, suckled beef cows were allotted to one of four treatment groups and injected with either saline or 500 ng LHRH every 2 h for 48 or 96 h, starting 21.4 ± .4 d after parturition. Two hours after the last injection of LHRH, cows were ovariectomized and 10 to 15 ovarian follicles per pair of ovaries were removed and categorized by diameter as small (1.0 to 3.9 mm), medium (4.0 to 7.9 mm) or large (≥8.0 mm). Injections of LHRH did not affect ($P>.10$) steroid levels in small follicles or numbers of gonadotropin receptors in small and medium follicles. Concentrations of progesterone in fluid of medium follicles increased 1.5-fold ($P<.05$) after 96 h of LHRH, whereas concentrations of estradiol and androstenedione were unchanged. In fluid of large follicles, concentrations of progesterone were fourfold greater ($P<.05$) in LHRH-treated than in control cows at 48 h, but by 96 h progesterone was twofold greater ($P<.05$) in control than LHRH-treated cows. In large follicles, concentrations of estradiol were unchanged ($P>.10$) after 48 h of LHRH injections but after 96 h estradiol was twofold greater ($P<.05$) in LHRH-treated than control cows. Increased concentrations of estradiol in large follicles coincided with increased numbers of binding sites for human chorionic gonadotropin (hCG) but not follicle stimulating hormone (FSH) in granulosa and theca. At 96 h, a greater proportion ($P<.05$) of LHRH-treated than control cows had at least one large follicle with concentrations of estradiol greater than progesterone (estrogen-active). The high concentrations of estradiol in estrogen-active follicles in LHRH-treated cows were associated with greater binding capacity of FSH to granulosa cells than found in estrogen-inactive (progesterone > estradiol in fluid) follicles. We suggest that increased capacity of large follicles to produce initially progesterone and bind hCG, then produce subsequently greater amounts of estradiol and bind FSH may be important steps in initiating ovulation in anovulatory cows treated with low-dose injections of LHRH.
(Key Words: Gonadotropin Releasing Hormone, Binding Site, Graafian Follicles, Steroids, Anestrus, Beef Cattle.)

Introduction

Increased frequency of pulsatile release of luteinizing hormone (LH) into blood occurs just before the pre-ovulatory gonadotropin surge in cyclic cattle (Rahe et al., 1980; Schallenberger et al., 1985) and in previously anovulatory cows after weaning (Walters et al., 1982b). Multiple, low-dose injections of LH-releasing hormone (LHRH; 500 ng/2 h) given for 4 d to anestrous, suckled beef cows induce LH and follicle stimulating hormone (FSH) pulses and(or) hasten time to first ovulation (Walters et al., 1982c; Spicer et al., 1986b). Therefore, use of low-dose injections of LHRH in anovulatory cows could be utilized to investigate initial intra-ova-

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rian events that presumably lead to selection of a single ovulatory follicle. The present study was undertaken to characterize changes in concentrations of serum estradiol, concentrations of steroids in follicular fluid, and numbers of follicular gonadotropin binding sites during administration of multiple, low-dose injections of LHRH to anovulatory, suckled beef cows.

Materals and Methods

Animals and Design. The 32 anovulatory, pluriparous beef cows were allotted to one of four treatment groups and received injections of either saline (.9% NaCl, 5 ml) or LHRH (500 ng/5 ml saline) every 2 h for 48 or 96 h as reported in Spicer et al. (1986b).

Serum Collection. Blood (20 ml) was collected via jugular cannulae 2 and 4 h before ovariectomy (Spicer et al., 1986b), and concentrations of progesterone in serum were analyzed as described (Convey et al., 1977). Intra- and inter-assay coefficients of variation were 3.7 and 10.6%, respectively for the serum progesterone radioimmunoassay. Concentrations of estradiol in serum were measured by radioimmunoassay using a 17β-estradiol ^{125}I-RIA kit[8] validated for bovine serum (Refsal, 1986). Intra- and inter-assay coefficients of variation were 5 and 21%, respectively. The sensitivity of the assay was approximately 2 pg/tube. The estradiol antiserum cross-reacts (at 50% displacement) 1.3, .4 and .02% with estrone, estriol and testosterone, respectively. Androstenedione and progesterone cross-reacts < .0005%. Secretory profiles of LH and FSH in serum from these cows have been reported (Spicer et al. 1986b).

Follicular Fluid and Tissue Collection. Ovaries were removed 2 h after the last injection of LHRH or saline as reported in Spicer et al. (1986b). Ten to 15 individual follicles were dissected from each pair of ovaries and their diameters classified as small (1.0 to 3.9 mm), medium (4.0 to 7.9 mm), or large (≥8.0 mm) as previously described (Spicer et al., 1986c). One of the large follicles from the 48-h LHRH group and two of the large follicles from the 96-h saline group ruptured during ovariectomy and their follicular fluids were lost. Follicular contents were collected by aspiration into syringes. Granulosa and thecal layers were removed and separated via blunt dissection from follicles 8 mm and greater in diameter, quickly frozen and stored in phosphate buffer saline (PBS) −20% glycerol (v/v) at −70 C. Small and medium follicles were diced and frozen as described previously. All follicular fluid and tissue were frozen within 3 to 5 h after ovariectomy.

Radioimmunoassay of Follicular Fluid Steroids. Concentrations of estradiol, progesterone and androstenedione were measured in follicular fluid using radioimmunoassays (Ireland and Roche, 1982). Respective intra- and inter-assay coefficients of variation were 12.9 and 20.3% for estradiol, 7.2 and 11.2% for progesterone, and 9.8 and 10.5% for androstenedione. Androstenedione was not measured in small follicles due to limited fluid volume.

Gonadotropin Binding Assays

Radioiodinations. Five micrograms of highly purified human chorionic gonadotropin (hCG; CR-119, 11,600 IU/mg) and 10 μg ovine FSH (Sairam-oFSH-S1390; 110 × NIH-FSH-S10) were radioiodinated using chloramine-T methods previously described (Spicer et al., 1981; Tonetta et al., 1985). Determinations of specific activity and maximum binding were routinely 20 to 50 cpm/pg and 15 to 40%, respectively.

Radioreceptor Assays. Numbers of hCG(LH) and FSH binding sites (expressed as cpm/μg DNA) were determined by saturation analyses (Ireland and Roche, 1982; Spicer et al., 1986a). Specific binding of ^{125}I-labeled hCG and ^{125}I-labeled oFSH was quantified in granulosa cells and thecal homogenates of large follicles. Binding of hCG(LH) and FSH was measured in homogenates of whole small and medium follicles. Binding assays within each size category were conducted using radiolabeled hCG and oFSH from a single iodination. Respective intra- and inter-assay coefficients of variation were 5.2 and 14.6% for hCG, and 5.6 and 13.1% for oFSH.

Statistical Analyses. Concentrations of progesterone and estradiol in serum were subjected to split-plot analysis of variance with "LHRH" and "duration of injections" as main plots, and "time of blood collection" as a subplot, and interactions. To test for significant changes in follicular sizes, concentrations of steroids in follicular fluid and amount of specific binding of gonadotropins to various-sized follicles during 48 and 96 h of either saline or LHRH injections

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data were subjected to a factorial analysis of variance with "LHRH" and "duration of injections" as main effects and interactions. All data were tested for nonhomogeneity of variances using Hartley's F_{max} test (Gill, 1978). Data showing heterogeneous variance (follicular fluid estradiol and progesterone) were analyzed after transformation to natural log (x + 1). This transformation did result in homogeneous variance. Mean differences were determined using Fisher's protected LSD mean test (Ott, 1977). Comparisons of follicular fluid steroid levels among small, medium and large follicles were made using Bonferroni's t-test (Gill, 1978).

To test for significant differences in concentrations of steroids in follicular fluid and amount of specific binding of gonadotropins to large follicles (≥8 mm) categorized as estrogen-active or estrogen-inactive, seven groups were formed (table 1) and a one-way analysis of variance and Fisher's protected LSD mean test (Ott, 1977) were conducted. Data on proportions of cows with estrogen-active follicles were compared among treatment groups using chi-square (Gill, 1978).

Results

Serum Progesterone. Mean concentrations of progesterone in serum at 2 and 4 h before ovariectomy did not differ (P>.10) among treatment groups and averaged .42 ± .04 ng/ml for LHRH-treated cows and .42 ± .03 ng/ml for control cows.

Serum Estradiol. Concentrations of estradiol in serum were slightly higher (45%; P>.10) at 2 and 4 h before ovariectomy in both 96-h saline- and LHRH-injected cows as compared with values after 42 to 48 h of injections (5.5 vs 3.8 pg/ml). Average concentrations of estradiol were slightly greater (18%; P>.10) in LHRH- vs saline-treated cows at 2 and 4 h before ovariectomy (5.9 vs 5.0 pg/ml).

Size of Follicles. Average (± SE) diameters of small (n = 162), medium (n = 146) and large (n = 46) follicles dissected from ovaries were 3.2 ± .1, 5.2 ± .2 and 11.0 ± .7 mm, respectively, and did not differ (P>.10) among treatment groups.

Follicular Fluid Progesterone. Mean concentrations of progesterone in fluid of small follicles ranged from 139 to 207 ng/ml and were not affected (P>.10) by LHRH. Progesterone in medium follicles was not affected by LHRH at 48 h (figure 1A). At 96 h, however, progesterone in medium follicles was 1.5-fold greater (P<.05) than at 48 h in LHRH-treated cows but remained unchanged in saline controls. Concentrations of progesterone in large follicles were fourfold higher (P<.05) in LHRH-treated than

TABLE 1. COMPARISONS AMONG FOLLICULAR FLUID (FF) STEROIDS AND GONADOTROPIN BINDING SITES IN GRANULOSA CELLS (GC) AND THECAL CELLS (TC) OF LARGE FOLLICLES (≥8 MM) CATEGORIZED AS EITHER ESTROGEN-ACTIVE (EA) OR ESTROGEN-INACTIVE (EI) AFTER 48 OR 96 H OF EITHER LHRH (L) OR SALINE (S) INJECTIONS IN SUCKLED BEEF COWS[a]

Follicle group	n[b]	Average diameter, mm	FF estradiol	FF progesterone	GC binding of ^{125}I-hCG	GC binding of ^{125}I-oFSH	TC binding of ^{125}I-hCG
			ng/ml FF		cpm/μg DNA		
48S-EI	6	8.8 ± .3[c]	11 ± 5[c]	81 ± 18[c]	829 ± 161[c]	1,183 ± 366[ce]	292 ± 64
48S-EA	6	10.9 ± .7[de]	146 ± 36[d]	51 ± 6[c]	582 ± 75[c]	1,928 ± 284[c]	448 ± 52
48L-EI	11	9.8 ± .5[cd]	12 ± 5[c]	413 ± 131[d]	1,550 ± 341[d]	696 ± 185[de]	298 ± 49
48L-EA	6	11.0 ± .9[de]	157 ± 42[d]	59 ± 9[c]	802 ± 203[c]	1,120 ± 298[ce]	280 ± 42
96S-EI[f]	10	9.8 ± .7[cd]	9 ± 4[c]	370 ± 141[d]	594 ± 114[c]	570 ± 174[d]	196 ± 43
96L-EI	6	10.0 ± .6[cd]	20 ± 10[c]	319 ± 129[d]	1,965 ± 627[d]	583 ± 177[d]	467 ± 224
96L-EA	7	12.5 ± .6[e]	208 ± 35[d]	73 ± 9[c]	1,881 ± 329[d]	1,008 ± 176[ce]	596 ± 203

[a]EA = Estradiol concentration > progesterone concentration in FF; EI = progesterone concentrations > estradiol concentration in FF.

[b]Number of follicles.

[c,d,e]Means (± SE) that do not have a common superscript within a column differ (P<.05).

[f]Only one follicle was EA in 96-h saline-injected group and was deleted from the table.

control cows after 48 h of injections (figure 1B). However, at 96 h concentrations of progesterone in large follicles were fivefold greater ($P<.05$) than at 48 h in control cows but were unchanged ($P>.10$) in LHRH-treated cows.

Follicular Fluid Androstenedione. Treatment with LHRH did not affect ($P>.10$) concentrations of androstenedione in fluid of either medium or large follicles. Concentrations of androstenedione averaged 25.6 ± 5.2 ng/ml in medium follicles and 13.0 ± 2.3 ng/ml in large follicles ($P<.05$) collected at 48 and 96 h after injections.

Follicular Fluid Estradiol. Compared with saline, LHRH injections for 48 or 96 h did not affect ($P>.10$) concentrations of estradiol in fluid (pooled mean ± SE) of small (6.8 ± .6 ng/ml) or medium (11.1 ± 2.5 ng/ml; figure 2A) follicles. In contrast, concentrations of estradiol in large follicles were 2.3-fold greater ($P<.05$) at 96 h than at 48 h in LHRH-treated cows, but decreased 74% in control cows during the same interval (figure 2B). Large follicles contained 7- to 12-fold greater ($P<.05$) concentrations of estradiol (79.8 ± 21.7 ng/ml) than small or medium follicles.

At 48 h, numbers of cows with at least one large follicle with concentrations of estradiol greater than progesterone (estrogen-active) were similar (chi square, $P>.10$) between LHRH- and saline-treated cows (figure 2B). At 96 h, a greater ($P<.05$) proportion (seven of nine) of LHRH-treated cows had large estrogen-active follicles as compared with control cows (one of seven).

Concentrations of estradiol in serum of individual cows were correlated ($r=.59$, $P<.05$) with average concentrations of estradiol in fluid of large follicles removed from the same cow less than 4 h later.

Gonadotropin Binding Sites in Follicular Tissue. Specific binding of ^{125}I-labeled hCG or ^{125}I-labeled oFSH to pooled homogenates of small follicles did not differ ($P>.10$) between control and LHRH-treated groups at either 48 or 96 h (data not shown). Average cpm of ^{125}I-labeled hCG and ^{125}I-labeled oFSH per μg DNA ranged from 350 to 500 and from 300 to 425, respectively.

Luteinizing hormone-releasing hormone did not affect ($P>.10$) gonadotropin binding in medium follicles (data not shown). Average cpm ^{125}I-labeled hCG and ^{125}I-labeled oFSH per μg DNA ranged from 525 to 725 and from 163 to 220, respectively.

In large follicles, binding of hCG(LH) to theca and granulosa did not differ ($P>.10$) between LHRH- and saline-treated cows at 48 h (figure 3 and 4A). However, at 96 h binding of hCG(LH) to theca and granulosa was 4.6- and 3.7-fold greater ($P<.05$), respectively, in LHRH- than in saline-treated cows. Histological examination of theca showed that contamination by granulosa cells was less than 10%.

Specific binding of ^{125}I-labeled oFSH to granulosa of large follicles was twofold greater ($P<.10$) in control than in LHRH-treated cows

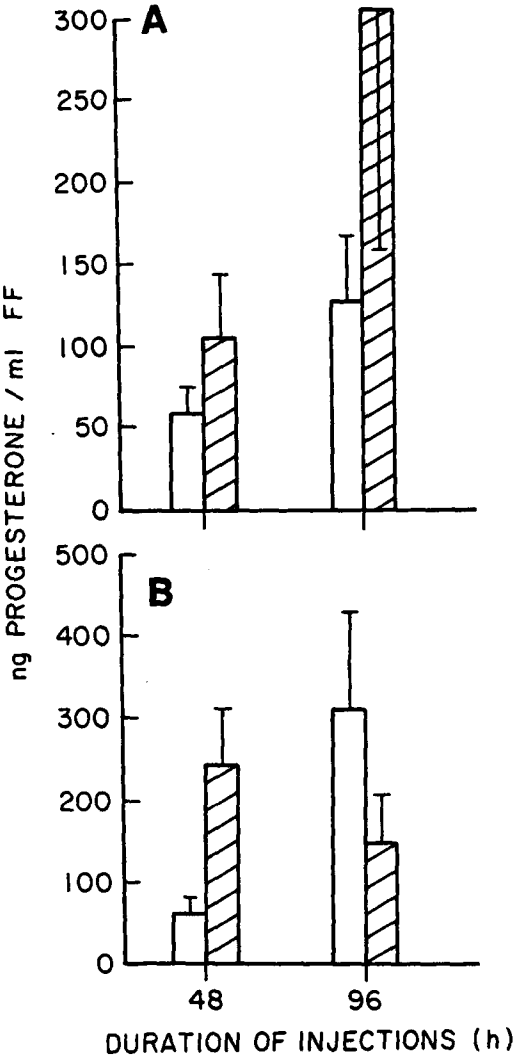

Figure 1. Concentrations of progesterone in follicular fluid (FF) of medium (A) and large (B) follicles after 48 or 96 h of either LHRH (▨) or saline (▭) injections.

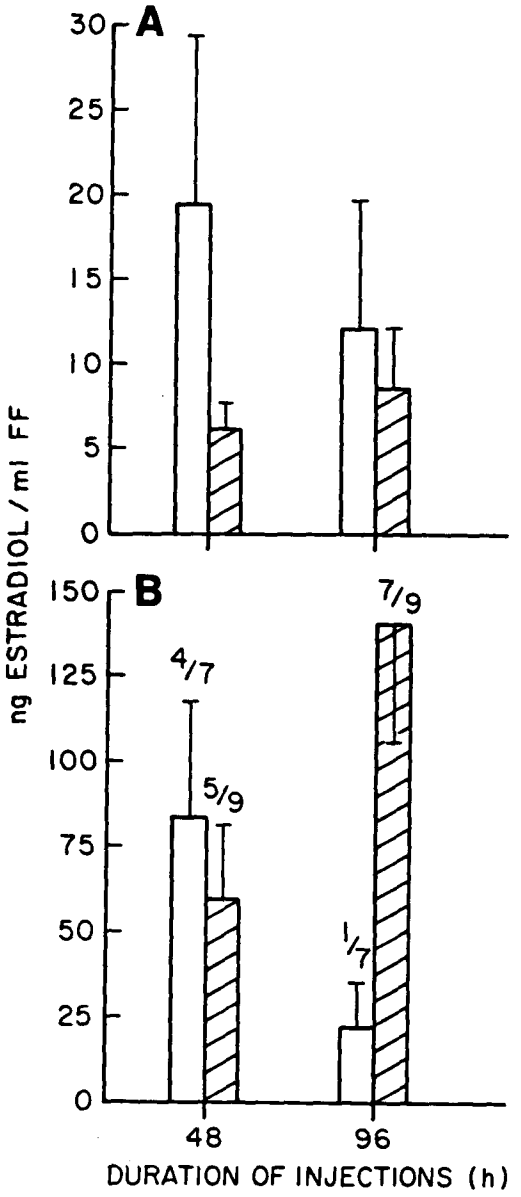

Figure 2. Concentrations of estradiol in follicular fluid (FF) of medium (A) and large (B) follicles after 48 or 96 h of either LHRH (▨) or saline (□) injections. Numbers above bars are numbers of cows with at least one large estrogen-active (estradiol > progesterone in follicular fluid) follicle per total number of cows.

at 48 h (figure 4B). However, binding of oFSH to granulosa was similar between control and LHRH-treated cows after 96 h (figure 4B).

To evaluate further relationships among follicular gonadotropin binding and fluid steroid levels, large follicles collected at 48 and 96 h were divided into seven groups: estrogen-inactive follicles from 48 or 96 h saline-injected cows; estrogen-inactive follicles from 48 or 96 h LHRH-injected cows; estrogen-active follicles from 48 h saline-injected cows; and estrogen-active follicles from 48 h or 96 h LHRH-injected cows (table 1). For LHRH-treated cows, estrogen-inactive (EI) follicles had similar concentrations of fluid estradiol and progesterone, and similar hCG(LH) and FSH binding between 48 and 96 h; in contrast, estrogen-active (EA) follicles had increased hCG(LH) binding to granulosa cells between 48 and 96 h, whereas fluid estradiol and progesterone and FSH binding remained constant. Binding of FSH to granulosa cells in EA follicles from LHRH-treated cows was greater than in EI follicles (table 1). For saline-injected cows, EI follicles (48S-EI and 96S-EI, table 1) had similar concentrations of estradiol and hCG(LH) binding to granulosa and theca at 48 and 96 h; however, fluid progesterone increased while FSH binding decreased in these same follicles. At 48 h, fluid estradiol in EA follicles from saline-injected cows (48S-EA) was greater than in EI follicles (48S-EI, table 1). All EI follicles were similar

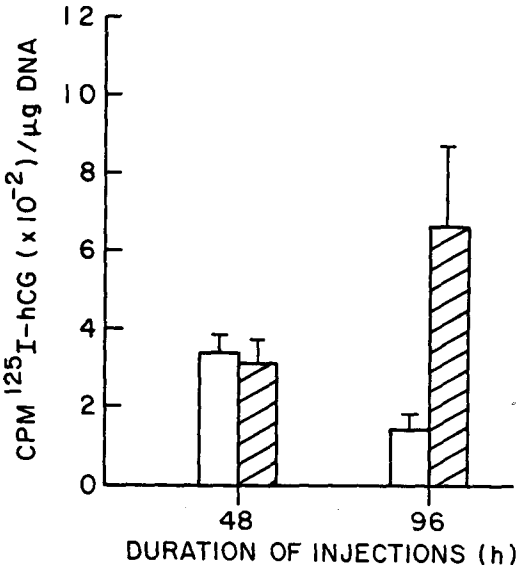

Figure 3. Specific binding of ^{125}I-labeled hCG (^{125}I-hCG) to thecal homogenates of large follicles after 48 or 96 h of either LHRH (▨) or saline (□) injections.

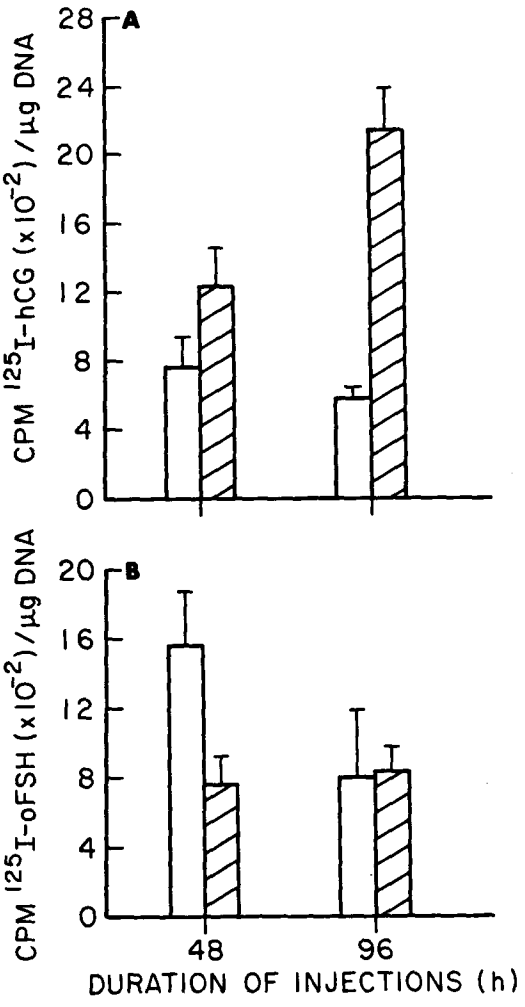

Figure 4. Specific binding of ^{125}I-labeled hCG (^{125}I-hCG; A) and ^{125}I-labeled oFSH (^{125}I-oFSH; B) to granulosa cells of large follicles 48 or 96 h of either LHRH (▨) or saline (□) injections.

in all variables measured except that greater progesterone levels existed at 48 h in LHRH-treated cows (48L-EI), and greater binding of hCG(LH) to granulosa cells existed in follicles from LHRH- vs saline-injected cows at both 48 and 96 h (48L-EI + 96L-EI vs 46S-EI + 96S-EI, table 1).

Concentrations of LH and FSH in serum (reported in Spicer et al., 1986b) were similar in cows with EA or EI follicles in saline-injected groups (data not shown). However, three of nine saline-injected cows without EA follicles had no definable LH or FSH pulses (Spicer et al., 1986b), whereas all five cows with EA follicles had definable LH and FSH pulses.

Discussion

Increased frequency of pulsatile LH release into blood occurs just before the pre-ovulatory gonadotropin surges in cattle (Rahe et al., 1980; Walters et al., 1982b; Schallenberger et al., 1985). However, the acute ovarian response to increasing pulsatile release of LH and FSH has not been studied in detail. Accordingly, a better understanding of the initial intra-ovarian events that lead to selection and eventual ovulation of a single follicle is of physiological interest. Coupled with results from a companion paper (Spicer et al., 1986b), the present study suggests that in the presence of unchanging follicular size and numbers, dramatic increases in estradiol in fluid of large follicles occurred coincidently with increased numbers of hCG (LH) binding sites in theca and granulosa of these same follicles. Perhaps these changes within large follicles are critical to preovulatory dominance of a single follicle. Increased capacity of follicles to bind hCG(LH) also has been observed during pre-ovulatory follicular development in cyclic cattle (Ireland and Roche, 1982; 1983b; Staigmiller and England, 1982; Walters et al., 1982b), sheep (Webb and England, 1982a,b), pigs (Stouffer et al., 1976) and rats (Uilenbroek and Richards, 1979).

Weaning calves from postpartum cows also increases numbers of hCG(LH) but not FSH binding sites in ovarian follicles prior to ovulation (Walters et al., 1982a,b). When comparing overall means we also observed an increase in hCG(LH), but not FSH binding sites, in large follicles from cows injected with LHRH. However, comparing EA and EI follicles collected after 96 h of LHRH injections, numbers of FSH receptors and not LH receptors were higher in EA than EI follicles. Thus, pooling estrogen-active and estrogen-inactive follicles may have masked changes in previous studies.

Hormonal stimuli for increased production of estradiol by large follicles after 96 h of LHRH injections cannot be discerned from our study because overall mean concentration of LH in serum remained unchanged in spite of increasing LH pulses. Moreover, overall mean concentration of FSH increased less than 20%

with increased FSH pulses (Spicer et al., 1986b). The inability to interpret such results of the present study raises an important question: Is the overall mean concentration of a given hormone more important than the secretory pattern of the hormone for eliciting its response from the target organ? The answer is unknown, but several reports suggest that pattern of secretion and not overall concentration of LH may be more important for inducing estradiol secretion by rat ovaries (Peluso et al. 1984a,c; Urbanski and Ojeda, 1985). Whether the increase in estradiol in fluid of large follicles is a result of or a cause of the increase in numbers of hCG(LH) binding sites in theca and granulosa also cannot be determined from the present study. Interestingly, when large follicles collected from LHRH-injected cows were categorized by estrogen to progesterone ratios, the major observed difference between high and low estradiol-containing follicles was not in number of hCG(LH) binding sites, but rather a difference in number of FSH binding sites in granulosa cells. In hypophysectomized rats, injections of either estradiol or LH can increase hCG(LH) and FSH binding sites in granulosa cells collected from antral follicles as long as FSH is given coincidently (Richards et al., 1978; Richards, 1980). Thus, the combination of increased estradiol production and FSH secretion (Spicer et al., 1986b) may have induced the increase in numbers of FSH binding sites observed in EA follicles. Since an increase in the number of hCG(LH) binding sites and not FSH binding sites was observed in EI follicles collected from cows injected with LHRH as compared with saline-injected cows, perhaps increased numbers of LH receptors is the intial intra-follicular event that precedes increased estradiol production and FSH binding during development of an estrogen dominant follicle.

Although concentrations of estradiol in fluid of individual follicles reflect the capacity of follicles to synthesize and secrete estradiol (Channing, 1980; England et al., 1981; Hillier et al., 1981; McNatty, 1982), dramatic increases in estradiol concentrations within large follicles may be reflected as only small increases in peripheral serum. In fact, after 96 h of LHRH injections, concentrations of estradiol in serum increased only 18% as compared with saline injections even though estradiol within large follicles was nearly sixfold greater in LHRH- vs saline-treated cows. Nonetheless, a significant correlation existed between estradiol in serum and follicular fluid. Increased estradiol secretion has been induced previously with low-dose injections of LHRH in anovulatory cows (Walters et al., 1982c) and sows (Cox and Britt, 1982).

The increase in proportion of cows with one large estrogen-active follicle (estradiol> progesterone in follicular fluid; Ireland and Roche, 1982) observed between 48 and 96 h of LHRH treatment most likely reflects increased numbers of healthy (non-atretic) follicles (Ireland and Roche, 1982; 1983a,b; Bellin and Ax, 1984). Whether this increase in EA follicles is due to change in function of pre-existing large follicles or to development of new follicles cannot be determined from our study. However, pulsatile, but not tonic, exposures of LH to rat ovaries in vitro has been suggested to "rescue" follicles from atresia (Peluso et al., 1984b).

In summary, results of the present study indicate that increased progesterone production precedes estradiol production from large follicles in cows injected with LHRH, suggesting that follicular enzymes responsible for converting progesterone to estradiol may be inadequate 21 d after parturition in suckled beef cows. In addition, increased estradiol production by large follicles is associated with increased numbers of hCG(LH) and FSH binding sites in theca and granulosa of cows injected with multiple, low-doses of LHRH. Collectively, the present studies suggest that initial intra-ovarian events that lead to selection of a single ovulatory follicle initially involve increased progesterone production and capacity to bind hCG(LH), and then an increased capacity to produce estradiol and bind FSH in large follicles.

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